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(54) Title: DIRECT HOMOGENEOUS ASSAY

(57) Abstract

Method and test kit for determining the presence of a bindable substance, e.g. antigen or antibody, in a test sample in excess of a predetermined amount and the quantitative measurement of any such excess.

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DIRECT HOMOGENEOUS ASSAY

FIELD OF THE INVENTION

The present invention is directed to the qualitative and quantitative determination of specific binding substances. The invention enables the presence or amount of one substance of any specific binding pair of substances to be determined by means of its complementary binding substance. In particular, the present invention is useful for determining whether a specific binding substance is present in a test sample in excess of a predetermined amount. The invention also permits quantitative measurement of any excess of the specific binding substance present in the test sample above a predetermined amount. Although the present invention has a variety of applications, it is particularly useful in the determination of immunochemical binding substances in biological fluids, for example, the detection or quantification of antigen or antibodies in human serum.

PRIOR ART

A number of different types of immunoassays are currently in use for the determination of the components of immunochemical binding pairs, e.g. antigens and antibodies, in various biological fluids. In general, an immunoassay, involves the determination of an immunoreactive substance, either directly or indirectly, by means of an immune reaction between the immunoreactive substance and a labeled form of its immunospecific conjugate or other receptor system.

Even substances that are not immunogenic by themselves, such as haptens, can be determined by immunoassay if they are bound to larger carrier substances which are capable of inducing antibody to the lower molecular weight substance. Immunoassays are useful in the detection of immune reactions in blood serum and are also employed in a number of immunohistochemical methods performed on tissue.

Immunoassays may be carried out with all of the immunochemical binding substances in solution, or with the immunoreactive substance or its immunospecific conjugate affixed to a solid support, such as glass or plastic tubes, beads, or microtiter plates. The latter is known as a solid-phase immunoassay. Solid-phase immunoassay is widely used due to its simplicity of performance and the ease with which the labeled complex may be separated from the unreacted, labeled antigen or antibody.

One type of solid-phase immunoassay involves a direct non-competitive binding technique for determination of immunochemical binding substances. In performing an immunoassay of this type to determine antibody, for example, antigen is immobilized on a solid support and then contacted with the test sample containing the antibody to be determined. Thereafter, a second antibody which is labeled and reacts specifically with the first antibody is added to the test sample. Because the second antibody is labeled the presence of the first antibody in the sample may be determined. By means of this technique, commonly known as a "sandwich technique", specific antibody can readily be detected in blood serum.

Solid phase immunoassays are also commonly

performed as competitive binding assays, based upon competition between labeled and an unlabeled forms of an immunoreactive substance for binding sites on the immunospecific conjugate. In order to determine the amount of antigen present in a test sample using a competitive assay, the test sample is usually mixed with labeled antigen, and then contacted with the corresponding antibody bound to a solid support, with the labeled and unlabeled antigen competing for antibody binding sites. The test sample is then separated into a liquid phase and a solid phase and the relative amount of labeled antigen present in either phase is quantitatively determined.

Most competitive binding immunoassays operate on the principle that an immunoreactive substance present in a test sample and a labeled form of the same substance, are attracted with essentially equal affinity to a solid support bearing the immunospecific conjugate. Therefore, the labeled and unlabeled forms of the immunoreactive substances become linked to the support in amounts proportional to the relative amounts of each substance in the sample. For example, if a test sample were to contain 90% of unlabeled antigen and 10% of labeled antigen, and the two substances were permitted to compete for a limited number of binding sites on the corresponding insolubilized antibody, the ratio of unlabeled antigen to labeled antigen becoming bound to the antibody would be 9:1.

It is also known to conduct solid-phase immunoassays by the saturation technique, in which a portion of immobilized immunospecific conjugate is saturated with the immunoreactive substance being determined in the test sample, and thereafter the

remaining unbound immunospecific conjugate is bound to a quantity of the labeled form of the immunoreactive substance being detected.

The direct, competitive and saturation techniques used in performing solid-phase immunoassays each permit quantitative determinations of immunoreactive substances by way of calculations based on the extent of binding of the labeled reagent employed in the assay.

Radioisotopes, enzymes and various chromophoric substances are commonly employed as labels in the above-described immunoassays. Radioisotopes provide a readily measurable signal which permits the results of the assay to be determined directly. Immunoassays employing such labels are generally characterized by exceptional sensitivity and accuracy.

Enzymes have been proposed as labels for immunochemical binding substances, especially in assays intended for home use. A notable advantage of the enzyme labels is that enzyme activity is detectable by the naked eye or by inexpensive detection equipment, such as a colorimeter. In performing an immunoassay using an enzyme-labeled reagent, the enzyme activity is measured by using a suitable substrate for producing an enzyme-catalyzed reaction, typically involving production or extinction of a colored compound whose light adsorption may be readily determined either qualitatively or quantitatively. Immunoassays employing enzyme labels are commonly referred to as enzyme-linked immunoabsorbant assays (ELISA).

It is known in the prior art to implement the ELISA procedure using a so called "test strip" or "dip stick" technique. These assays employ a bibulous

substrate or carrier, which may be a natural or synthetic polymeric material, but is typically a cellulosic material, such as filter paper, to which is affixed the immunospecific conjugate of the immunoreactive substance being determined. The enzyme label may be linked to the immunospecific conjugate bound to the support, or with a component of the immunochemical binding pair in the test sample. These assays are intended to eliminate the manipulative steps, e.g. washing and incubation which are normally involved in the standard ELISA technique, thereby reducing the chance of error, so as to permit use by untrained personnel. Moreover, because the procedure may be conducted rapidly and gives a visual result, these assays are desirable for use in a doctor's office and in the home.

An ELISA-type assay employing a test strip having antibodies bound thereto is described in U.S. Patent No. 4,168,146. This assay is based on the aforementioned "sandwich technique". In use, the antibody-bearing test strip is immersed in the test sample suspected of containing the antigen to be determined. After the test sample migrates along the test strip, and any antigen present in the test sample reacts with the antibodies bound to the test strip, the test strip is contacted with a solution containing antibodies linked to an enzyme label and the enzyme activity is determined, as described above.

Another test strip-type assay, employing a combination of enzymes, is disclosed in U.S. Patent No. 4,435,504. The test strip is in the form of an immunochromatograph having a porous support permitting solvent travel, a plurality of a specific binding pair

substance (e.g. antibody) and an enzyme, the latter two components being non-diffusively and uniformly bound to the support to define an immunosorbing zone. Also employed in this assay is an enzyme-labeled specific binding pair member (e.g. enzyme-labeled antigen), which binds to the immunosorbing zone in relation to the portion of the immunosorbing zone in which the target substance being determined (e.g. antigen) is bound, to define a border related to the amount of target substance present in the test sample. It is disclosed that the enzyme label is preferably related to the enzyme on the immunochromatograph, such that the substrate of one is the product of the other. In practice, the immunochromatograph is contacted with the test sample for a time sufficient for the test sample to migrate across the immunosorbing zone. The immunochromatograph is also contacted with the labeled specific binding pair substance in a solvent medium, so that the labeled specific binding pair substance binds in the immunosorbing zone in relation to the target substance bound thereon. Thereafter, the immunosorbing zone is contacted with a development solution containing a substrate for the enzyme label to produce a measurable signal, and the distance of the aforesaid border from one end of the immunochromatograph is determined, in order to quantify the amount of target substance in the test sample.

Another example of an assay employing a test strip having enzyme-labeled immunochemical binding substance present thereon is found in U.S. Patent No. 4,446,232. When used for the determination of antigen, the test strip comprises a first zone containing antigens and enzyme-linked antibodies which are capable of

immunochemcially reacting with the antigens and a second zone containing a substance capable of undergoing a color forming reaction with the enzyme label linked to the antibodies, thereby to indicate the presence of the antibodies in the second zone. The antibodies are diffusively bound in the first zone, such that they will pass from the first zone to the second zone upon reaction with antigens from the test sample migrating through the first zone, but will not diffuse through the first zone in the absence of migrating antigens. In practice, the test strip is contacted with a test sample suspected of containing the target antigen for a time sufficient for the test sample to migrate along the test strip, and the presence or absence of any color change is observed as an indication of the presence or absence of the target antigen in the test sample. It is disclosed that this assay may be adapted for quantitative determinations by providing it with multiple, separate regions, each containing a different amount of immobilized reference antigen, the position of the color change being dependent on the concentration of the antigen in the test sample.

Although the test strips proposed heretofore for implementing the ELISA technique may provide a rather uncomplicated and relatively rapid procedure for the qualitative and/or quantitative determination of immunochemical binding substances, these prior art assays do not enable the determination of the target substance in excess of a predetermined amount, or the quantitative measurement of any excess of the target substance beyond the predetermined amount.

SUMMARY OF THE INVENTION

The present invention provides a simple and convenient method and test kit for determining the presence of a bindable substances in a test sample in excess of a predetermined amount and the quantitative measurement of any such excess. In carrying out the method, there is provided an array of complementary binding substance which is capable of binding to the bindable substance and having a predetermined binding capacity for the bindable substance. The test sample and the array of complementary binding substances are contacted for a time sufficient to allow binding of bindable substance in the test sample to the complementary binding substance. Thereafter, a carrier medium containing a given amount of labeled bindable substance is contacted with the array of complementary binding substance. The given amount of labeled bindable substance is such that, when added to the predetermined amount of bindable substance being determined, the binding capacity of the complementary binding substance is substantially filled. The absence or significant presence of unbound labeled bindable substance is then determined to differentiate whether or not the bindable substance is present in the test sample in excess of the predetermined amount. Since the binding capacity of complementary binding substance would be substantially filled by the sum total of the predetermined amount of bindable substance being determined (if present in the test sample) and the given amount of labeled bindable substance that is brought into contact with the array of complementary binding substance, the significance presence of unbound labeled bindable substance indicates that bindable

substance was present in the solution in excess of the predetermined amount. The amount of unbound labeled bindable substance can also be determined quantitatively to give a measurement of the degree to which the bindable substance in the test sample exceeds the predetermined amount. The array of the present invention is described as a homogeneous array, in that no separation of bound and free specific binding pair substance is required.

The test kit of the present invention comprises the substances and devices necessary to perform the above-described method. The test kit includes: 1) an array of complementary binding substance on a support, the array having a predetermined binding capacity for the bindable substance being determined; and 2) labeled bindable substance in an amount sufficient, when added to the predetermined amount, to substantially fill the binding capacity of the complementary binding substance.

The method and test kit of the present invention provides a simple, rapid and reliable method for determining chemical inbalances in various biological and other fluids. The present invention may be used to particular advantage in medical diagnosis and in detecting drugs in relatively low concentrations in body fluids.

DESCRIPTION OF THE INVENTION

The present invention is directed to the qualitative and quantitative determination of one substance of a specific binding pair, consisting of a bindable substance and its complementary binding substance. In the description of the invention that

follows, the binding pair substance being determined is referred to as the bindable substance, although in practice, either of the binding pair substances could be determined using the method and test kit of the present invention.

The term "bindable substance" refers to any substance which reacts with a complementary binding substance based on the mutual specific binding affinity between the two substances. The term "bindable substance", as used herein, includes, but is not limited to proteins, hormones, both polypeptides and steroids, carbohydrates and glycoproteins. Representative examples of specific binding pairs are antigens and their antibodies, haptens and their antibodies, hormones and their receptors, vitamins and their receptors and toxins and their receptors, determinations of all of which are within the scope of the present invention.

The term "complementary binding substance" refers to the specific binding partner of the bindable substance being determined. The complementary binding substances used in the practice of this invention are generally underivitized, which means that they are not bound to, or otherwise conjugated with, any other chemical moiety. The complementary binding substance is provided in the form of an array on a suitable support. The support can be in the form of a glass or plastic capillary, a paper strip, polymer beads, a column packed with a suitable matrix, or the like. There are many methods well known to those skilled in the art for affixing a complementary binding substances to such supports. Such methods employ, for example, covalent bonding as well as other types of affixation,

such as absorption. Any method may be employed that avoids significant diffusion of the bound substance. The complementary binding substance is preferably applied to the support as a one-dimensional lattice, by which is meant a linear array of binding sites such that target substance binding and saturation begins at one end and proceeds uniformly to the other. This may be achieved by arranging the binding substance on a three dimensional support whose length is significantly greater than width, height or cross sectional area. Alternatively, the binding component may be affixed to a two dimensional support whose length is significantly greater than its width.

The "predetermined amount" employed as the standard in carrying out the present invention may vary depending on the particular type of bindable substance in question and the nature of the assay being conducted. In the case of biological fluids, for example, there exists established levels of various components considered to be the normal or average amounts, which are commonly referred to as "clinical norms". In applying the present invention as an immunoassay, the predetermined amount of bindable substances to be determined would generally be the clinical norm.

By way of example, the following table presents the clinical norm for some of the various components of human serum:

Component	Normal Value
J-1 Antitrypsin	210-510 mg/dl
Ceruloplasmin	20-35 mg/dl
Cholesterol	150-280 mg/dl
Creatinine	0.8-1.2 mg/dl

Component	Normal Value
Folate	5-21 ng/ml
Gastrin	30-200 pg/ml
IgG	720-1500 mg/dl
IgA	90-325 mg/dl
IgM	45-150 mg/dl
Lipids	450-100 mg/dl
Albumin Proteins	4.0-5.0 g/dl
Globulin Proteins	2.0-3.0 g/dl
Quinidine	4-6 mcg/ml
Serotonin	0.1-0.32 mcg/ml
Thyroxine (total)	4-11 mcg/dl
Thyroxine (free)	0.8-2.4 ng/dl
Uric Acid	3.0-7.0 mg/100 ml
Vitamin A	0.15-0.6 mcg/ml
Vitamin B_{12}	330-1025 pg/ml

In addition to the array of complementary binding substance, the method of the present invention involves the use of a labeled form of the bindable substance being determined, or a substance having the same binding affinity for the complementary binding substance as the bindable substance being determined. Suitable labels include radioisotopes, enzymes and chromophoric substances, the latter including dyes which absorb light in the U.V. or visible region of the electromagnetic spectrum, and fluroescent or phosphorescent substances. Appropriate methods for linking of any of the aforesaid labels to a bindable substance of the type described above are well known to those skilled in the art. An important requirement of the method of labeling the bindable substance is that it not sterically hinder or interfere with the reactive sites of either component of the specific binding pair. Radioactive or phosphorescent labels rarely interferes with the stereochemical properties of chemical substances to which they are linked. In a system in which the target bindable substance is antigen or

hapten, with the array of complementary binding substance consisting of antibody to said antigen or hapten, the labeled bindable substance may consist of antigen covalently linked to an enzyme. Of course, in this embodiment, the enzyme must be linked to the antigen or hapten in such a way that the affinity of the antigen or hapten for the antibody is not impaired, or otherwise diminished. Enzymes that have exhibits enzymatic activity after in conjugated form include, catalases, perioxidases, glucuronidases, glucosidases, galactosidases, urease and oxidoreductaces, such as glucose oxidase and galactose oxidase.

An important aspect of the present invention is that a known relationship exists between the value of the predetermined amount (i.e. the clinical norm in the case of an immunoassay) of the bindable substance being determined, the amount of label, and the number of available binding sites of the complementary binding substance on the array. Since the bindable substance and the label are the same substance, or are substances having substantially the same binding affinity for the complementary binding substance on the array, any bindable substance present in the test sample competes equally with the labeled bindable substance for the available binding sites on the complementary binding substance. Because the array of complementary binding substance has a known binding capacity for the bindable substance, which is substantially filled by the clinical norm of bindable substance and the given amount of labeled binding substance used in carrying out the method, the presence of bindable substance in the test sample in excess of the clinical norm may be readily determined. Thus, if more than the clinical

norm of bindable substance is present in the test sample, there will not be sufficient binding sites on the array to accommodate all of the unlabeled and labeled bindable substance and and an excess, or "spillover", of both unlabeled and labeled bindable substance will result. Since the unlabaled and labeled bindable substance are competing for the available binding sites on the array of complementary binding substance with equal affinity, the spillover will virtually always contain labeled bindable substance, since, as noted above, the unlabeled and labeled bindable substance will be bound in proportion to the relative amount of each that is contacted with the array of complementary binding substance. Accordingly, the significant presence of unbound labeled bindable substance in the spillover differentiates whether or not the bindable substance in the test sample exceeds the established norm. As used herein, the expression "significant presence" refers to an amount which is two-three-times background noise, or error level in the test system.

In practice, a test sample believed to contain bindable substance to be determined is contacted with the array of complementary binding substance for a time sufficient to allow binding of the bindable substance to the complementary binding substances to occur. The available binding sites on the complementary binding substance are filled to the extent of the bindable substance present in the test sample. The array is then contacted with labeled bindable substances in a carrier medium. Suitable carrier mediums include aqueous or other polar solvents, such as ether or alcohol for biological systems. Non-polar solvents may

be used in other systems where appropriate. The carrier medium may be buffered to the desired pH range, and a detergent may be added to reduce non-specific binding. If the test sample contained the clinical norm of bindable substance, then the labeled bindable substance will substantially fill the remaining available binding sites of the complementary binding substance on the array.

Instead of the sequential procedure just described, the labeled bindable substance may be incorporated in the test sample for contact with the array of complementary binding substance simultanesouly with the bindable substance being determined. According to the this procedure, the labeled and unlabeled bindable substances compete directly for available binding sites on the array. In this procedure the test sample serves as the carrier medium.

In performing the method of the present invention for the determination of a particular antigen in a sample of human serum, the number of sites provided on the array of conjugate antibody substantially equals the amount of antigen corresponding to the clinical norm and the amount of labeled antigen which is added in carrying out the method. Since the number of available binding sites on the array is known, and the clinical norm of the antigen in question is also known, the amount of labeled bindable substance employed may be varied to achieve the desired substantial filling of the array. For example, if the clinical norm for a certain antigen fills 60 binding sites on an array of antibody, then an array having, for example, 120 available binding sites may be provided and used in

conjunction with an amount of labeled antigen which will substantially fill the remaining 60 binding sites. Of course, if it is desired to change the number of binding sites, one must only change the amount of label used to still achieve the intended result. After the array is contacted with both the test sample and the labeled antigen (either sequentially or simultaneously, as noted above), if more than 60 units of antigen are present in the test sample, then all of the labeled antigen will not be bound to the array. The absence or significant presence of label remaining in the test sample (or carrier medium) after contact with the array thus indicates whether the particular antigen in question is present in the serum in excess of the clinical norm.

The present method is adaptable for use in a physician's office or at home, in the form of a simple test kit. The test kit includes a suitable support to which is affixed an array of complementary binding substance having a predetermined number of binding sites, along with a predetermined amount of labeled bindable substance. The amount of labeled bindable substance would depend on the clinical norm of the substance being tested for and the number of sites on the array. If such a test kit employed enzyme as the label, a visible signal would be produced in the spillover thus obviating specialized detection equipment. Such an assay could be performed on body fluids, such as saliva or urine by relatively untrained personnel.

In accordance with the present invention, it is also possible to quantitatively measure the amount by which bindable substance in a test sample exceeds an

established norm. When the above-described procedure is performed on a test sample of serum containing more than the clinical norm of a particular antigen, the effluent obtained after contacting the test sample or carrier medium with the array contains unbound labeled and unlabeled antigen. The amount of unbound labeled antigen in the effluent may then be measured. In the case of an enzyme label, the enzymatic activity of the effluent may be determined, for example, by measuring color intensity in a colorimeter. If a radioactive label is used, the radioactivity of the effluent may be measured. Once the amount of labeled antigen in the effluent test solution or carrier medium is determined, the amount of antigen initially present in the sample solution can be easily calculated, since binding of the labeled and unlabeled antigen occurs proportionally to the concentration of labeled and unlabeled antigen initially present. Specific measurements may be made using a standard curve, as is well known in the art.

For example, where a column is employed containing an array of antibodies providing 300 binding sites, and the clinical norm for the antigen in the test sample occupies 80 of the binding sites, 220 units of labeled antigen are added to the test solution. Whenever more than the clinical norm of antigen is present in the test sample, a corresponding increase in the amount of labeled antigen remaining in the effluent test solution will be detectable. Table I, below, shows the theoretical amounts of labeled antigen which would be "spilled over" in the effluent when 220 units of labeled antigen are mixed with gradually increasing quantities of unlabeled test antigen and run on an array of antibody having a 300 binding site

capacity.

Units Test Antigen Added (Ag)	Total Antigen In "Spill Over" 300-(220+test Ag)	Labeled Antigen in "Spill Over" 220/(220+test ag) x spilled Ag
80	0	0
100	20	13.8
120	40	25.8
140	60	36.6
180	100	55
200	120	62.9
220	140	70
300	220	93
400	320	113
500	420	128

A plot of effluent activity (labeled antigen in "spill over") plotted against test antigen present in the sample solution (in excess of 80 units) provides a curve which is substantially linear. Once a table of this nature is prepared for a given antigen and its antibody it is thereafter only necessary to measure the enzyme activity of the labeled antigen in the "effluent" to find the amount of antigen present in the test sample in excess of the clinical norm.

In yet another embodiment of the present invention, it is contemplated that the array of complementary binding substance may be affixed to a solid support with an indicator substance present on

the solid support adjacent to the array. For example, if it were desired to test a sample of serum for an antigen component normally present in an amount of 80 units, an array of antibody may be provided having a binding capacity of, for example, 300 units of antigen. 220 units of enzyme labeled antigen would then be added to the test sample or to a separate carrier medium. Adjacent to the array of 300 sites, on a separate portion of the same solid support, there would be affixed an indicator substance, such as a substrate which produces a color change under the influence of the enzyme label. The sample solution would then be contacted with the array so as to fill the 300 available binding sites before reaching and contacting the indicator substance. If the amount of antigen present in the sample solution were not greater than the clinical norm of 80 units, then substantially all of the antigen, both labeled and unlabeled, would be bound to the array of antibody, and none of the antigen would reach the indicator portion of the support. If, however, the test sample were to contain more than the clinical norm of 80 units of antigen, the labeled antigen would reach the indicator substance on the support and produce an visible signal, by means of a color change.

This latter embodiment of the invention enables quantitative determination of the amount by which the bindable substance in a test sample exceeds the clinical norm. Referring to the preceding example in which an indicator substance is employed, the area of the indicator substance on the solid support which undergoes reaction with the label of the immunoreactive substance relates to the amount of the substance

present in excess of the established norm. The area of the indicator substance undergoing reaction with the label bears essentially the same functional relationship to the excess as the data reported above in Table I.

It is preferred to employ a porous cellulosic material, e.g. an elongated paper strip, as the solid support in the embodiment just described, so as to permit the test sample to diffuse readily along the support by capillary action. Immunoreactive substances and indicator substances such as enzymes may be bound directly to a paper support by methods well known in the art.

The following examples further describe the manner and process of making and using the present invention and set forth the best mode currently contemplated for carrying out the invention, but are not to be construed as limiting the invention.

EXAMPLE I

This example describes the making of a test device for use in the determination of DNP and DNP-derivitized proteins, in accordance with the present invention.

Rabbit antiserum directed against DNP was prepared by immunization of rabbits with dinitrophenylated random sequence polypeptide composed of 40% glutamic acid, 30% lysine and 30% alanine. The synthetic polypeptide had 10% of its lysines conjugated with DNP. Using precipitation analysis with DNP_{10}Bovine Serum Albumin ($DNP_{10}BSA$), the titer of the antiserum with respect to DNP was determined to be 2.1 mg. antibody/ml. of serum.

Antiserum was coupled to Sepharose 4B by the sodium carbonte/Sepharose/cyanogen bromide activation method. Prior to coupling to Sepharose, the antiserum was decomplemented by adsorption with rabbit BSA anti-BSA precipitates, dialyzed against buffer (0.1 M $NaHCO_3$) and centrifuged in a microfuge. To achieve

uniform coupling all reagents were mixed rapidly and resulted in an adsorbent slurry.

Micro-columns containing the adsorbent slurry were constructed using 20 microliter disposable micro-pipettes as the body of the micro-columns. A 10 to 15 cm. length of 1.5 mm. internal diameter plastic tubing was fitted to the bottom of the micro-pipette to serve as an outflow tube. Prior to fitting the plastic tubing over the end of the micro-pipete a small piece of cotton or a fragment of tissue was inserted into the end of the plastic tubing. When fitted over the end of the micro-pipette, the tissue or cotton material butted against the bottom of the micro-pipette forming a barrier through which adsorbent could not pass. To faciliate filling of the micro-columns the micro-pipette was fitted with a funnel constructed by taking a standard laboratory plastic pipette tip and slicing the end to allow insertion of the micro-pipette.

The micro-columns were first filled with phosphate buffered saline (PBS) containing 1% bovine serum albumin and incubated for 1 hour to reduce non-specific adsorption to the sub-nanogram levels. The columns were filled by introducing an appropriate charge of the adsorbent slurry. Each column was brought to a bed volume of 20 microliters by removing excess adsorbent using a needle and syringe. The columns were 69 to 72 mm. high and could be filled with consistency to within 0.5 mm. Therefore, bed volumes could be controlled to within 0.7%.

The micro-columns were calibrated using radioactive $DNP_{1.8}BSA$. The $DNP_{1.8}BSA$, which was carefully determined by appropriate

absorbance measurements at 280 and 360 nanometers, was labeled with iodine 125 by the Iodogen method. An activity of 6 microCuries/Microgram was achieved and it was found, as result of binding to mini-columns (100 to 300 microliters), that the labeled protein was undamaged by labeling, since the appropriate amounts of labeled protein were bound when diluted with unlabeled protein.

To determine the capacity of the micro-columns, saturating levels of labeled antigen were delivered into each of seven of the micro-columns. The labeled antigen was delivered into the micro-columns in volumes of 20 through 80 microliters. After loading the columns with the labeled antigen, 5 column volumes of 1% BSA/PBS were passed through the columns. The average number of counts bound was reliable with a deviation of approximately 5%, regardless of the number of counts introduced to the column. The capacities of the columns is set forth in Table II.

Table II

sample column#	counts delivered	counts bound
1	6,500,000	570,460
2	10,000,000	610,310
3	8,500,000	585,700
4	2,000,000	620,120
5	1,200,000	605,360
6	800,000	578,200
7	650,000	592,450

The average number of counts bound was 595,660. From the specific activity of the diluted antigen, this corresponds to 460 nanograms of antigen.

EXAMPLE II

This example describes an experiment in which labeled DNP/BSA was added to the test sample for

simultaneous delivery to the test device with unlabeled DNP/BSA.

Mini-columns of 460 nanogram capacity were constructed as described in Example I. Samples containing known amounts of unlabeled DNP/BSA were mixed with 200 nanograms (260,000 counts per minute) of labeled DNP/BSA and applied to the micro-columns. After delivery of the test samples seven column volumes of BSA/PBS were passed through each column, and the effluent was collected in a counting vial and counted. The results are shown in Table III, two trials being run of each sample.

Table III

unlabeled antigen added (nanograms)	Trial 1	Trial 2
0	7024	6842
240	6785	7210
260	7320	7428
280	11960	12200
300	21406	22780
360	45500	48200
450	76635	79200
550	105149	98460

From the results of these experiments it can been seen that background counts are collected for 0,240, and 260 nanogram levels, while the collected values climb rapidly above background once the column capacity is exceeded (starting at 280 ng).

EXAMPLE III

The experiment of Example II was performed using a sequential rather simultaneous delivery of the labeled and unlabeled DNP/BSA to the test device.

The test samples were delivered to the column first in a volume of 30 microliters, followed by 20 microliters of buffer and then by 200 nanograms (260,000 counts per minute) of the labeled antigen.

The results of this experiment are set forth in Table IV.

Table IV

unlabeled antigen (nanograms)	Trial 1	Trial 2
0	5840	6540
240	7013	5923
260	7550	7450
280	27575	28360
300	51400	55290
360	130600	136400
450	266010	257380
600	264800	255600

The results of this experiment show that enhanced sensitivity can be obtained using this procedure, as the radioactivity of the effluent is substantially increased. This is because no initial dilution with unlabeled antigen occurs. It is noted that a small margin of error exists, since more counts were present in the effluent than were introduced in the case of two of the samples. However, this error may be considered experimentally acceptable.

EXAMPLE IV

This example describes a experiment employing a different calibration system which was found effective for measuring lower levels of antigen. For this experiment, rabbit antiserum was first diluted with normal rabbit serum 1:5 to lower the level of antibody on the absorbant. Calibration of the micro-column was carried out as described previously and showed that the 20 microliter columns had a total capacity for 120 nanogram of antigen. In carrying out this experiment, 60 nanograms of labeled DNP-BSA (80,000 counts per minute) were mixed with known samples and passed through the columns. The effluents were collected and counted as previously described. The results of this experiment are given in Table V.

Table V

unlabeled antigen (nanograms)	Trial 1	Trial 2
0	1290	-
20	980	-
40	1123	-
60	1230	-
80	10800	11560
100	20400	23500
140	28700	29500
200	45000	41980

Although the method and test kit of the present invention has been described with specific reference to use as an immunoassay, the inventions has other utilities as well. For example, the invention may be used for the determination of specific binding pair substances, (e.g. DDT and naturally occurring DDT-binding substances), present as hazardous

contaminants in ground water in excess of standards established by federal regulation. Thus, if the method and test kit of the present invention were used for assaying ground water for compliance with federal regulatory requirements, the predetermined amount would be the standard established by the relevant regulation.

While the method and test kit of the present invention have been described herein in terms of certain preferred embodiments, various other embodiments may be apparent to those skilled in the art. Therefore, the invention is not limited to the embodiments actually described, but is capable of variation and modification without departing from the spirit and scope of the appended claims.

What is claimed is:

1. A method for determining whether or not one of a pair of substances having mutual specific binding affinity, and consisting of a bindable substance and a complementary binding substance, is present in a test sample in excess of a predetermined amount, said method comprising:

a.) providing an array of complementary binding substance having a predetermined binding capacity for said bindable substance;

b.) contacting said array of complementary binding substance with:

(i) said test sample, for a time sufficient for any bindable substance present in said test sample to bind to said complementary binding substance; and

(ii) labeled bindable substance in a carrier medium, the amount of said labeled bindable substance being sufficient, when added to said predetermined amount, to substantially fill the binding capacity of said complementary binding substance, said array of complementary binding substance and said labeled bindable substance being in contact for a time sufficient for said labeled bindable substance to bind to said complementary binding substance; and

c.) determining the absence or significant presence of unbound labeled bindable substance to differentiate whether or not said bindable substance is present in said test sample in excess of said predetermined amount.

2. A method according to claim 1, wherein said carrier medium for said labeled bindable substance is said test sample.

3. A method according to claims 1 or 2, wherein the test sample is human serum, the bindable substance is antigen, and the complementary binding substance is antibody specific to said antigen.

4. A method according to claim 3, wherein the predetermined amount of antigen is the clinical norm for a given volume of human serum.

5. A method according to claim 3, wherein the antibody is arrayed on an elongated substrate.

6. A method according to claim 5, wherein said antibody is arrayed on the interior surface of a capillary tube.

7. A method according to claim 5, wherein said antibody is arrayed on a paper strip.

8. A method according to claim 3, wherein the array comprises polymer beads having antibody bound thereto.

9. A method according to claims 1 or 2, wherein the label is selected from the group of an enzyme, a radioisotope, or a chromophoric substance.

10. A method according to claims 1 or 2, wherein

the unbound labeled bindable substance is qualitatively determined.

11. A method according to claim 10, wherein the label is an enzyme, the test sample is collected after contact with said array of complementary binding substance and the unbound enzyme-labeled bindable substance is determined by adding to the collected test sample a substrate which changes color under the influence of said enzyme.

12. A method according to claim 10, wherein the unbound labeled bindable substance is determined by placing adjacent to said array of complementary binding substance an indicator substance responsive to the label of said labeled bindable substance, said test sample contacting said indicator substance after contacting said array of complementary binding substance, whereby the presence of any unbound labeled bindable substance in said test sample contacting said indicator substance is indicated by said indicator substance.

13. A method according to claims 1 or 2, wherein the unbound labeled bindable substance is quantitatively determined.

14. A method according to claim 13, wherein the label is an enzyme, the test sample is collected after contact with said array of complementary binding substance and the unbound enzyme-labeled bindable substance is determined by adding to the collected test

sample a substrate for said enzyme and measuring the enzyme activity in said solution.

15. A test kit for determining whether or not one of a pair of substances having mutual specific binding affinity, and consisting of a bindable substance and a complementary binding substance, is present in a test sample in excess of a predetermined amount, said test kit comprising:

a.) a complementary binding substance capable of binding to said bindable substance, said complementary binding substance being arrayed on the surface of a support for contact with said test sample, said complementary binding substance having a predetermined binding capacity for said bindable substance; and

b.) labeled bindable substance in an amount which, when added to said predetermined amount, is sufficient to substantially fill the binding capacity of said complementary binding substance.

16. A test kit according to claim 15, further comprising an indicator substance for indicating the presence of unbound labeled bindable substance in the test sample after contact with said complementary binding substance.

17. A test kit according to claim 15, wherein said complementary binding substance is antibody and the labeled bindable substance is labeled antigen having specific binding affinity for said antibody.

18. A test kit according to claim 17, wherein the label is selected from the group of an enzyme, a radioisotope or a chromaphoric substance.

19. A test kit according to claim 16, wherein the label is an enzyme and the indicator substance is a substrate which changes color under the influence of said enzyme.

20. A test kit according to claim 15, wherein said support further comprises an indicator substance responsive to the label of the labeled bindable substance, said indicator being disposed on said support adjacent to said complementary binding substance.

21. A test kit according to claim 20, wherein the label is an enzyme and the indicator substance is a substrate which changes color under the influence of said enzyme.

22. As an article of manufacture, a solid support having affixed thereto a substantially one dimensional array of underivitized antibody providing a predetermined number of antigen binding sites and, adjacent to said array of antibody, a substrate for an enzyme capable of linking to antigen having specific binding affinity for said antibody, said substrate changing color under the influence of said enzyme.

23. An article of manufacture according to claim 22, wherein the solid support is elongated and the

array of antibody and the substrate are arranged lengthwise on said solid support.

24. An article according to claim 22, wherein said support is a paper strip.

25. An article according to claim 22, wherein said support is a capillary tube, and the antibody and substrate are arranged on the interior surface of said capillary tube.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00668

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4] G01N 33/50,53,538

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/4,7,805,810 436/518,527,530,531,534,808,809,810

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	US,A, 4,446,232 (LIOTTA) 1 May, 1984. See the entire document	22-25 1-21
A,P	US,A, 4,533,629 (LITMAN) 6 August 1985	
Y	US,A, 3,991,174 (GRUNDMAN) 9 November 1976, see column 2, lines 6-17	1-21
Y	US,A, 4,039,652 (ADAMS) 2 August 1977, see columns 3 and 4 and column 9 lines 62-64	1-21

* Special categories of cited documents: [15]

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IV. CERTIFICATION

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International Searching Authority [1]	**Signature of Authorized Officer [20]**
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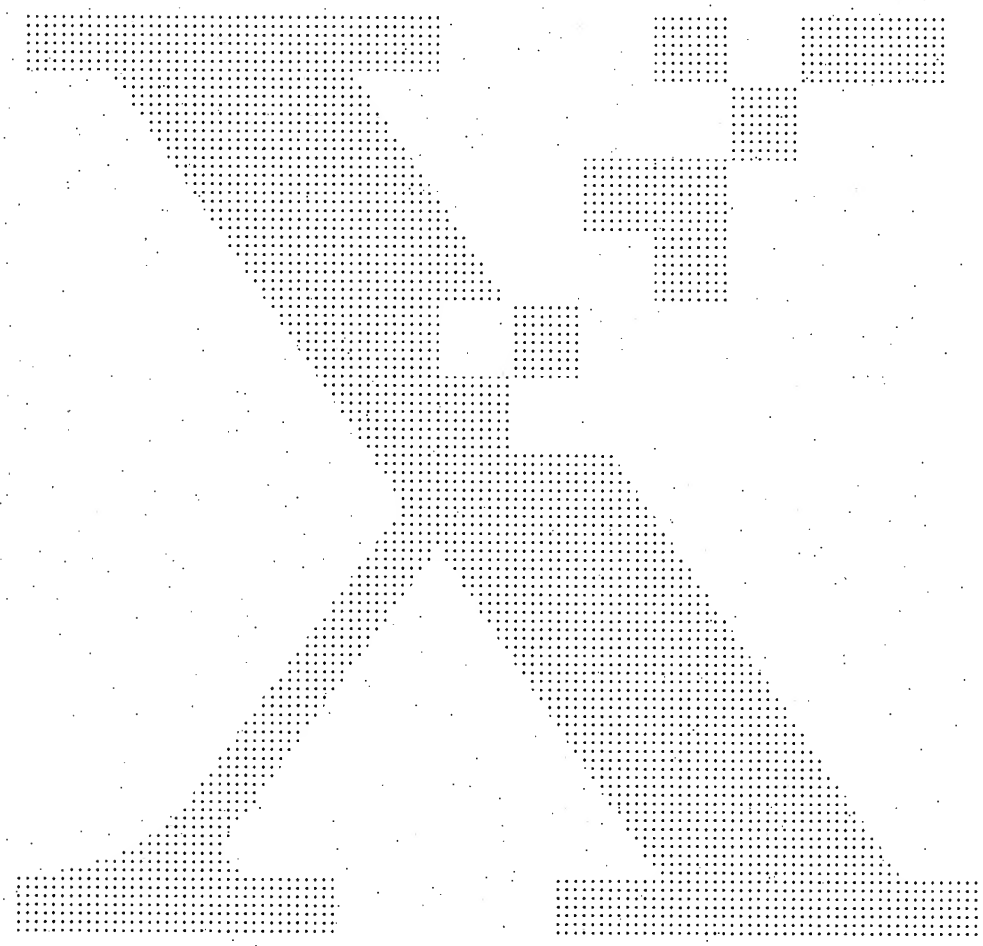

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(54) Title: ISOLATION OF FETAL CELLS FROM MATERNAL BLOOD TO ENABLE PRENATAL DIAGNOSIS

(57) Abstract

This invention relates to a method for the isolation of trophoblast (placental) cells from the blood of a pregnant mammal so as to provide the essential starting material, namely cells derived from the fetus, to enable genetic and/or biochemical information about the fetus to be obtained. In particular, this invention relates to the use of monoclonal antibodies specific for membrane protein markers on mammalian trophoblasts to isolate trophoblast cells from maternal blood. These cells may then be used to obtain fetal genetic and/or biochemical information early in pregnancy. The present invention is particularly relevant for detecting human fetal abnormalities.

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ISOLATION OF FETAL CELLS FROM MATERNAL BLOOD TO ENABLE PRENATAL DIAGNOSIS

This invention relates to a method for the isolation of trophoblast (placental) cells from the blood of a pregnant mammal so as to provide the essential starting material, namely cells derived from the fetus, to enable genetic and/or biochemical information about the fetus to be obtained. In particular, this invention relates to the use of monoclonal antibodies specific for membrane protein markers on mammalian trophoblasts to isolate trophoblast cells from maternal blood. These cells may then be used to obtain fetal genetic and/or biochemical information early in pregnancy. The present invention is particularly relevant for detecting human fetal abnormalities.

Currently, prenatal testing is carried out on fetal cells obtained by either amniocentesis or chorionic villous sampling (CVS). Amniocentesis is normally performed around 16 weeks of gestation. The procedure involves attendance by skilled personnel to insert a needle into the amniotic sac of the fetus and remove between 20-30 ml of amniotic fluid. The amniotic fluid contains fetal cells to allow subsequent tests to be performed. This method of obtaining fetal cells is associated with a risk of inducing a spontaneous abortion. In addition, if the subsequent genetic diagnosis of the fetus reveals an abnormality, the

prospect of a mid-trimester pregnancy termination is both psychologically stressful and associated with some physical risk to the mother.

Chorionic villus sampling also requires the involvement of skilled personnel to take a small biopsy from the placenta of an 8-12 week old fetus. Again this procedure has some risk of inducing a spontaneous abortion, although the early diagnosis of any chromosomal abnormality makes the procedure more attractive than amniocentesis. However, the need for skilled personnel and the possibility of inducing spontaneous abortion for both procedures means that current prenatal genetic assessments are made only on pregnant women who are deemed "at risk" of carrying a chromosomally defective fetus.

The method of the present invention provides a simpler procedure which involves obtaining blood from an arm vein of a pregnant mammal such as a pregnant woman and extracting fetal cells which are normally sloughed off from the placenta into the maternal circulation. No specialised expertise is required to obtain this blood sample and this non-invasive isolation of fetal cells negates any risk of inducing a spontaneous abortion. The blood may be taken around the same gestational time as for chorionic villus sampling hence the benefits of early diagnosis are gained.

Although the presence of trophoblast cells in maternal peripheral blood has been the subject of some debate, Goodfellow and Taylor (1982) have reported the extraction of trophoblast cells circulating in peripheral blood during pregnancy by use of differential centrifugation. Covone et al (1984) investigated the possibility of detecting trophoblast cells in the peripheral blood from women at various stages of

gestation by the use of monoclonal antibody H315 (Johnson et al, 1981), however in subsequent reports (Pool et al, 1987; Adinolfi et al, 1989), it was suggested that the isolation of H315-positive cells as a source of diagnostic material for antenatal diagnosis of fetal abnormalities is impractical. Recent data suggests that the frequency of fetal cells in the maternal circulation (from 12 weeks to 36 weeks gestation) is less than 1 in 100,000 (Adinolfi et al, 1989; Schwinger et al, 1989).

According to one aspect of the present invention, there is provided a method for the isolation of trophoblast cells from a blood sample of a pregnant mammal which method comprises contacting said blood sample with a binding-effective amount of an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells for a time and under conditions sufficient for said antibody to bind to target cells and then separating said cells bound by said antibody from said sample.

Another aspect of the present invention is directed to a method for obtaining fetal genetic and/or biochemical information in a pregnant mammal which method comprises isolating a blood sample from said pregnant mammal and contacting said blood sample with a binding-effective amount of an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells for a time and under conditions sufficient for said antibody to bind to said cells and then separating cells bound by said antibody from said sample and obtaining from the isolated cells genetic and/or biochemical information.

Yet another aspect of the present invention relates to a kit for the isolation of trophoblast cells from a blood sample of a pregnant mammal and optionally

for obtaining genetic and/or biochemical information about said cells comprising in compartmental form a first container adapted to contain an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells; optionally a second container adapted to receive and contain a blood sample from said pregnant mammal; and optionally a third container adapted to contain a means for obtaining from the isolated cells genetic and/or biochemical information.

In a preferred embodiment of the present invention, the mammal is a pregnant human female and one or more of the antibodies FD0161G or FD066Q or FD0338P is/are used.

Still yet another aspect of the present invention relates to the homogeneous or near homogeneous antigens, FD0161G or FD066Q protein and FD0338P protein, or their derivatives.

The present invention is further described with reference to Figure 1. This figure is a photographic representation of an analysis of products from PCR of trophoblast cells isolated from peripheral blood of pregnant women. Analysis was performed in a 15% (w/v) polyacrylamide gel. Tracks 1 & 8: DNA size markers, (pUC19/Hpa II digest; Top-button 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34 bp); tracks 2 & 3: trophoblast cells isolated from blood of two individual women carrying a male fetus; tracks 4 & 5: trophoblast cells isolated from the blood of two individual women carrying a female fetus; track 6: no DNA blank; track 7: control trophoblast cells derived from a full-term placenta of a male child.

The present invention is directed to a method for isolating trophoblast (placental) cells from a blood

sample of a pregnant mammal. The trophoblast cells so isolated are a convenient source of genetic and/or biochemical material from which fetal analysis can occur such as for potential fetal abnormality. The isolation of trophoblast cells is predicated on the use of antibodies, and in particular monoclonal antibodies, although not necessarily limited thereto, specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells. Accordingly, in one aspect of the present invention there is provided a method for the isolation of trophoblast cells from a blood sample of a pregnant mammal which method comprises contacting said blood sample with a binding effective amount of an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells for a time and under conditions sufficient for said antibody to bind to cells and then separating cells bound by said antibody.

For the purpose of exemplification only, the present invention is described using blood from a pregnant human female to isolate human placental cells. This is done, however, with the understanding that the present invention extends to all mammals. In extending to mammals other than humans, it may be necessary to alter the specificity of the trophoblast binding antibodies. The present invention, therefore, extends to all such antibodies as well as those, described herein, specific for human villous syncytiotrophoblast and non-villous cytotrophoblast cells.

By "binding effective amount of antibody" as used in the specification and claims herein is meant an amount of antibody sufficient to bind to the target cells and be used in the isolation of such cells. It is a preferred embodiment of the subject invention that the antibodies be coupled to a substrate such as magnetic polystyrene beads precoated with sheep anti-mouse IgG (Fc fragment)

serum (Dynabeads M-450, Dynal AS, Oslo, Norway). However, other substrates could be used such as a fluorescent chemical. The blood sample is contacted with an effective amount of beads, i.e. from about 2000 to 10,000 beads/ml of whole blood and preferably about 4000 beads/ml (i.e. about 10^5 beads per 25ml sample) and allowed to incubate at 4°C overnight. The beads with trophoblast cells attached via the specific trophoblast reactive antibody are removed using the cobalt-samarium magnet (Dynal AS). These methods represent an optimal protocol in terms of performance and/or convenience but may be subject to variations to suit the particular situation but which are still within the scope of the present invention which contemplates all such variations.

Accordingly, the present invention relates to a method for the isolation of trophoblast cells from a blood sample from a pregnant woman, which comprises contacting the blood sample with an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells, and subsequently separating cells bound by said antibody from said sample.

The fetal trophoblast cells so isolated from the maternal blood sample can then be assessed for their genetic and/or biochemical characteristics using known diagnostic techniques.

Either monoclonal or polyclonal antibodies may be used or combinations thereof for the step of separating trophoblast cells from the blood sample, however the use of a monoclonal antibody is preferred.

In a particularly preferred aspect of this invention, three antibodies have been used for the isolation of trophoblast cells. They are designated FD0161G (Mueller et al, 1987), FD066Q and FD0338P.

These antibodies are each a mouse monoclonal antibody secreted by an individual hybridoma cell line which grows indefinitely in tissue culture and can be stored frozen in liquid nitrogen. Further details of these monoclonal antibodies and the production thereof are provided herein. Each of these three monoclonal antibodies are of the heavy chain subclass, G, and light chain, kappa. Other sources of monoclonal antibodies are encompassed by the present invention.

The monoclonal antibodies FDO161G and FDO66Q, have apparent specificity for the same, or a closely associated epitope of a trophoblast membrane protein (hereinafter referred to as the "FDO161G/FDO66Q protein, or antigen") which resides on villous syncytiotrophoblast and non-villous cytotrophoblast cells of human first trimester and term placentas and invading non-villous cytotrophoblast cells in human decidua.

The protein has not been detected on villous cytotrophoblast cells using either the FDO161G or the FDO66Q antibody. It was detected on a restricted number of other human tissues, viz. thecal and granulosa cells of mature ovarian follicles, interstitial cells of testis and cells in the zona fasciculata/glomerulosa of adrenal cortex, but it was not detected on a wide range of other human tissues and cells, including peripheral blood cells and villous mesenchyma. These results are presented in Tables 1 & 2. The epitope of the membrane protein recognised by the FDO161G antibody (and the same, or closely associated epitope recognised by FDO66Q) was, however, detected on baboon and marmoset placentas. It was also present on cultured human first trimester trophoblast cells and a human choriocarcinoma line, JEG-3.

Table 1

MONOCLONAL ANTIBODY REACTIVITY ON HUMAN TISSUES

Mab clone:	FDO161G (FDO66Q)	FDO338P (FDO78/93P)
Tissue:		
6-12 wk Placenta	+++	+++
Term Placenta	++	++
Decidua	-	-
Endometrium (proliferative)	-	-
Myometrium	-	-
Ovary	+[1]	-
Testis	+[2]	-
Kidney	-	-
Liver	-	-
Spleen	-	-
Liver	-	-
Lung	-	-
Adrenal	+[3]	-
Pancreas	-	-
Skin	-	-
Striated Muscle	-	-
Thyroid	-	-
Pituitary	-	-
Stomach	-	-
Rectum	-	-
Colon	-	-

Notes:
1. Reactive with thecal cells in mature follicles and the corpus luteum.
2. Reactive with interstitial cells.
3. Reactive with cortex cells (zona fasciculata /glomerulosa).

Table 2

MONOCLONAL ANTIBODY REACTIVITY ON HUMAN CELL SUSPENSIONS

Cells:	Mononuclear Leukocytes	Granulocytes	T-ALL[1]	Granulosa Cells
Mab clone:				
FDO161G (FDO66Q)	-	-	-	+++
FDO338P (FDO78P) (FDO93P)	-	-	NT	-
FDO81C[2]	+++	+++	+++	+++

Notes:

NT Not tested.

1. T-acute lymphatic leukemia (JM-Line).

2. FDO81C reacts with all cells. Used as positive control.

The monoclonal antibody, FD0338P, detects an epitope of a protein ("FD0338P protein or antigen") which resides on the human trophoblast membrane. This epitope is expressed on villous syncytiotrophoblast of human first trimester and term placentas. The epitope was also present on invading, non-villous cytotrophoblast cells of human decidua but at much lower density. It was not detected on villous cytotrophoblast cells of human placentas or on villous mesenchyma. The epitope of this membrane protein recognised by the FD0338P antibody was not detected on a wide range of other human tissues and cells including peripheral blood cells and serum components. These results are presented in Tables 1 & 2. Although the epitope defined by FD0338P was not detected on baboon or marmoset trophoblast, a related glycoprotein analogue is present. This analogue can be identified by other monoclonal antibodies produced in the inventors' laboratory, viz. FD078P & FD093P, which are directed to other epitopes on the human FD0338P antigen.

The cell and tissue distribution of the trophoblast membrane proteins detected by FD0161G or FD066Q monoclonal antibodies, and FD0338P or FD078P or FD093P monoclonal antibodies are distinct from each other and from all other trophoblast antigens previously described.

Thus the monoclonal antibodies described by Johnson et al (1981) which react with syncytiotrophoblast also recognise decidual glands (H315) or lymphocytes (H316). The antibody NDOG1 (Sunderland et al, 1981) binds syncytiotrophoblast and cytotrophoblast and recognises a carbohydrate epitope. Trop. 1 and Trop. 2, described by Lipinski et al (1981) bind both syncytiotrophoblast and villous cytotrophoblast. The monoclonal antibody described by Loke and Day (1984) binds villous cytotrophoblast and endometrial glands

(Anderson et al 1987). Monoclonal antibodies FDO161G, FDO66Q and FDO338P are all distinct when compared with the specificities of antibodies submitted to a World Health Organisation-sponsored Workshop held in 1986 (Anderson et al, 1987).

Accordingly, in a preferred aspect of this invention, the method for isolation of trophoblast cells comprises contacting the maternal blood sample with an antibody, preferably a monoclonal antibody, specific for an epitope(s) of trophoblast membrane protein(s) and subsequently separating cells bound by said antibody(ies) from said sample. One of three monoclonal antibodies is preferred, viz. the antibodies FDO161G and FDO66Q, which bind to epitope(s) of a membrane protein antigen residing on villous syncytiotrophoblast and non-villous cytotrophoblast cells and the antibody FDO338P which binds to an epitope of a different membrane protein antigen residing on villous syncytiotrophoblast and to a lesser extent, on non-villous cytotrophoblast cells. Thus these antibodies produced in the inventors' laboratory are specific for two distinct membrane protein antigens, FDO161G/FDO66Q protein and FDO338P protein, described in detail below. Furthermore, it is within the scope of the present invention to include the use of more than one antibody in combination. Hence, one or more antibodies may be employed.

The protein antigens detected by the monoclonal antibodies FDO161G or FDO66Q and FDO338P have been prepared from human term placentas. The placental tissue is solubilised using a detergent (CHAPS) to release individual membrane proteins. Agarose (Sepharose) beads with either FDO161G or FDO66Q or the FDO338 antibody covalently attached are then added to the mixture. After suitable incubation the beads are recovered and respective antigen which is bound to the monoclonal

antibody, is then dissociated from the antibody using an acid solution

The FD0161G/FD066Q protein has been isolated from detergent-solubilised human term trophoblast membranes using affinity gel chromatography. It migrates as a single entity on sodium dodecyl-sulphate polyacrylamide gel electrophoresis having a molecular weight of 43 kiloDalton (kDa) for the dithiothreitol reduced protein. The unreduced protein migrates at a similar molecular weight indicating that the protein is not composed of polypeptide subunits. The protein was detected on the surface of cultured trophoblast cells by immunochemical analysis and on human ovarian granulosa cells by fluorescence analysis, indicating that it is a glycosylated protein.

The nature of the carbohydrate linkages to the polypeptide backbone of the FD0161G/FD066Q protein was determined using a combination of enzymatic and chemical cleavage. Using endoglycosidase F, an enzyme which cleaves high mannose, hybrid and complex glycans adjacent to the amino acid, asparagine (i.e. all N-linked carbohydrate), no reduction in molecular weight was observed. This indicates that the FD0161G/FD066Q protein contains no N-linked glycans. Using Trifluoromethanesulphonic acid, a chemical which cleaves both N- and O-linked glycans from the polypeptide backbone, a significant reduction in molecular weight (from 43kDa to 31 kDa) was observed. This indicates that the FD0161G/FD066Q protein contains only O-linked glycans (i.e. linked to either serine or threonine). Since each chain of oligosaccharide accounts for an approximate molecular weight difference of between 2000-4000 Da, it may be estimated that the FD0161G/FD066Q protein has between 3-6 oligosaccharide chains per molecule.

Amino acid sequencing of this protein antigen was performed by standard methods on both the N-terminus of the molecule and an internal segment. The latter was derived after proteolytic digestion with Protease V8 (from Staphylococcus aureus V8) in phosphate buffer. This serine protease cleaves on the carbonyl side of Glu and Asp residues under these conditions. The amino acid sequences were determined using an automated gas-phase sequenator and conventional Edman degradation chemistry. The following sequences were obtained:

N-Terminal Sequence of the FDO161G/FDO66Q protein Thr[1]-Gly-Trp-Ser-His-Leu-Val-Thr-Gly-Ala[10]-Gly-Gly-Phe-Leu-Gly-Gln-(Arg)-Ile-(Ile)-(Arg)[20]-Leu-(Leu)-Val-Lys-(Lys)[25].

Internal Sequence was obtained from Protease V8 digest of FDO161G/FDO66Q protein

-Phe[1]-(X)-Leu-Arg-Leu-Glu-Ser-Arg-(X)-Ser[10]-Phe-Pro-Leu-(Ser)-(X)-Met-Tyr-(X)-Ile[19].

Assignments were considered unambiguous unless in round parentheses (hereinafter designated Y). The assignments marked (X) indicate that no amino acid was released and may possibly be O-linked glycosylation sites (i.e. amino acid may either be Ser or Thr).

Both of the above sequences have been compared with the following Data bases: NBRF Standard Data Base; NBRF Auxiliary Data Base; Kyoto Data Base; Swiss Data Base; Newat Data Base (Total: 4525 sequences - 1,116,976 amino acid residues). A correlation coefficient was set at 0.75. Only two proteins from the data bases exceeded this level, i.e. Paper Wasp (Polistes jadwigae) for the N-terminal sequence and Alpha-2u-globulin precursor from mouse for the internal, Protease V8 digest, fragment. Since the correlation coefficients were only 0.764 (over 14 amino acids) and 0.779 (over 9 amino acids)

respectively, it may be concluded that the protein defined by FDO161G or FDO66Q monoclonal antibodies has not previously been sequenced.

On SDS-polyacrylamide gel electrophoresis, the FDO338P antigen prepared by affinity chromatography (as described above) migrates as a series of molecular weight entities ranging from 30 kDa through to the largest, 67 kDa. The same bands are seen under both reducing and non-reducing conditions, indicating that the protein does not possess either inter- or intrachain disulphide bonds. The two largest and most abundant species, namely, the 63 and 67 kDa bands have been isolated to apparent homogeneity by electro-elution from the SDS-polyacrylamide gel. The protein eluted from FDO338P gel was shown to contain carbohydrate residues. The carbohydrate was removed with Trifluoromethanesulphonic acid, resulting in reduction of molecular weight of the protein to 30 kDa. Assuming that each oligosaccharide chain contributes between 2000-4000 Da to the apparent molecular weight on SDS-PAGE gels, it is estimated that the FDO338P glycoprotein has between 8-16 oligosaccharide chains. The nature of the oligosaccharide linkage to the polypeptide chain was investigated using endoglycosidases F and H. Endoglycosidase F cleaves both complex and high mannose N-linked (i.e. to asparagine residues), but not O-linked (i.e. to serine/threonine residues) oligosaccharides. Cleavage with this enzyme reduced the molecular weights of the two major bands from 63 and 67 kDa to 43 and 48 kDa respectively. Endoglycosidase H cleaves only high mannose-type N-linked oligosaccharides. No reduction in molecular weight was seen after treatment with this enzyme indicating an absence of high mannose chains. Thus the FDO338P glycoprotein appears to have between 3-6 O-linked oligosaccharide chains and 5-10 complex N-linked oligosaccharide chains.

Accordingly, the present invention extends to homogeneous or near homogeneous FD0161G/FD066Q protein and FD0338P protein and/or their derivatives. By derivatives is meant any alteration such as addition, deletion and/or substitution to the amino acid and/or carbohydrate sequence or components of said proteins and extends to proteins or parts thereof associated with various molecules (e.g. lipids, other proteins etc.). All such proteins are encompassed by the present invention together with any antibodies, monoclonal or polyclonal, made thereto. By "homogeneous or near homogeneous" is meant a preparation at least 70% pure relation to other protein and preferably greater than 80%-90% pure.

The isolation procedure for the fetal trophoblast cells in the blood sample in accordance with the present invention may involve, for example, the use of murine monoclonal antibody FD0161G or FD066Q or FD0338P coupled to a substrate such as magnetic beads. One such substrate which is commercially available and which has been found to be effective comprises uniform, magnetic polystyrene beads with affinity purified sheep anti-mouse IgG, covalently bound to the surface. Each monoclonal antibody (MAb) is secreted by an individual hybrid cell line which was produced by fusing mouse myeloma cell (P3X63-Ag8-653) with a mouse spleen cell producing the antibody. The individual hybrid cell lines of interest were thus immortalised. On growing in culture, each cell line secretes the respective MAb into the culture medium which can then be collected and used as a source of antibody. This culture supernatant is used to coat the magnetic beads using the procedure described in Example 2. To extract trophoblast cells from the blood of pregnant women, use is made of the ability of the MAb FD0161G, FD066Q or FD0338P, to bind specifically to trophoblast cells in the maternal blood and not to any

other circulating blood cell. Thus MAbs bind specifically to cells and/or cell fragments which carry the trophoblast membrane proteins defined by each MAb as described above. The beads, with trophoblast cells attached, can then be extracted from the blood cell suspension using a magnet. In an alternative isolation method, the MAbs may be labelled with a fluorescent label, and trophoblast cells bound by the fluorescent-labelled MAbs identified and removed from the sample in a fluorescence-activated cell sorter.

The trophoblast cells isolated by either method can be examined to obtain genetic and/or biochemical information. A number of techniques are known for obtaining genetic information in particular for determining fetal sex and genetic abnormalities. One such technique involves the use of segments of nucleic acid as probes or primers.

Nucleic acid probes are hybridized to the nucleic acid of the isolated trophoblast cells. This method is based on the fact that two complementary strands in a deoxyribonucleic (DNA) double helix can be separated by denaturation and then reannealed (hybridized) under conditions where the hydrogen bonding of base pairs is favoured. Therefore, if the correct complementary nucleic acid sequence is present on the nucleic acid strands of the isolated trophoblast cells, resulting in hybridization of the nucleic acid probe, an appropriate signal can be measured. Since the number of fetal trophoblast cells is very low, a technique of amplifying the signal is desirable. For example, through the use of DNA primers, the polymerase chain reaction, can be used. In this process, the specific nucleic acid sequence targeted through the DNA primers is copied many times by an enzyme, Taq Polymerase. This enables detection of the amplified product by electrophoresis on agarose or

polyacrylamide gels. Visualisation of the DNA can be carried out using ultraviolet light after staining with ethidium bromide, or, through detection of radio-labelled product, from radio-labelled nucleotides. Furthermore, the isolated trophoblast cells can be useful for biochemical analysis.

To demonstrate the utility of the trophoblast cell isolation from blood using the monoclonal antibodies FD0161G or FD066Q or FD0338P and the magnetic polystyrene beads, the polymerase chain reaction (PCR) has been used to distinguish between cells of male or female origin. To determine the sensitivity of detection, peripheral blood lymphocytes were used. Using Y chromosome specific primers a signal has been reproducibly obtained from as few as 6 male lymphocytes after 30 amplification cycles of PCR. An equivalent number of female cells taken through the same procedure yields either no detectable amplification product (more common) or a small amount of a slightly smaller sized product readily distinguished from that of male cells.

Blood samples (approx 25ml) were obtained from pregnant women about to undergo chorionic villous biopsy for detection of fetal genetic abnormalities. Fetal trophoblast cells were isolated from the samples using magnetic beads coated with Mab FD0161G or FD0338P as described. The isolated cells attached to the beads were processed through the PCR using the Y-chromosome specific primers. This enabled prediction of the sex of the fetus. Of eleven samples processed, the prediction was confirmed in eleven by routine chromosomal analysis of the chorionic villous sample carried out independently in an unrelated laboratory ($P < 0.0005$). This result demonstrates the utility of the methodology, thus:

1. That the antibodies FDO161G or FDO66Q and FDO338P which define two separate human trophoblast membrane proteins can be employed to isolate fetal cells.

2. That the cells isolated are of fetal origin since male cells were identified in seven cases.

3. That the cells isolated can subsequently undergo procedures which can detect genetic markers with appropriate probes.

Another aspect of this invention is in the detection of genetic abnormalities and/or fetal sex by examination of chromosomes after culture of the isolated cells. For example, this would enable detection of the common genetic disorder, Down's syndrome, for which it is less likely that a genetic probe will become available in the near future.

The cells isolated by the magnetic bead technique can be placed into plastic culture dishes and induced to divide by the addition of growth factors derived from other cultured cells. Preferred cell cultures are those of decidual cells obtained during elective termination of pregnancy, and human granulosa cells obtained during the procedure of egg collection for In Vitro fertilization. Cell free supernatants from these cell cultures are added at a suitable concentration to the isolated trophoblast cell suspensions. Division of cells can be arrested at an appropriate time by the addition of known reagents, e.g. colchicine, then the chromosomes analyzed by standard cytogenetic diagnostic techniques.

The present invention also extends to a kit for the isolation of trophoblast cells from a blood sample of a pregnant mammal and optionally for obtaining genetic and/or biochemical information about said cells

comprising in compartmental form a first container adapted to contain an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells; optionally a second container adapted to receive and contain a blood sample from said pregnant mammal; and optionally a third container adapted to contain a means for obtaining genetic and/or biochemical information from the isolated trophoblast cells.

The antibody in the first container is either a monoclonal antibody or a polyclonal antibody or a combination thereof, and even more preferably FD0161G or FD066Q or FD0338P and/or combinations thereof. It is a preferred embodiment that the monoclonal antibody is first bound to a substrate such as the magnetic polystyrene beads discussed above.

The following examples describe in detail the production of the monoclonal antibodies to the human trophoblast membrane proteins of this invention and the use of these antibodies to demonstrate the utility of the invention. The methods used in this demonstration are included by way illustration and not limitation of the subject invention, it being recognized that various modifications are possible within the scope of the invention.

EXAMPLE 1

PRODUCTION OF MONOCLONAL ANTIBODIES

a. Preparation of Syncytiotrophoblast

First trimester placentas were obtained from elective terminations of apparently healthy pregnancies performed by aspiration at 6-10 weeks gestation. Clotted

blood and any adherent decidua were carefully dissected from the placentas. Syncytiotrophoblast was isolated by gently teasing the placentas through a 250-mesh sieve. The sheets of syncytiotrophoblast, being significantly larger than contaminating cells, readily sediment at unit gravity in Earle's Balanced Salt Solution (Flow Laboratories, Sydney, Australia). After sedimentation for approximately 2 min, the supernatant was decanted and the cells resuspended in fresh solution. This washing procedure was performed three times, then the cells were either used for immunization in mice or placed into culture. The success of trophoblast isolation was confirmed by the synthesis of human chorionic gonadotrophin in culture after three days incubation. Human chorionic gonadotrophin concentration was measured with a solid phase two-site immunoradiometric assay (Hybritech, California, USA).

Balb/c mice were immunized intraperitoneally with 0.5ml of trophoblast cell suspension. The immunogen was given at weekly intervals for six weeks following a three week delay after the primary injection.

b. Preparation of choriocarcinoma cell suspension

The choriocarcinoma line, JEG-3, was obtained from the American Type Culture Collection. Cells were cultured in 6-well dishes in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, L-Glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in a humid atmosphere of 5% CO_2 in air. When the cultures were confluent, the cells were scraped from 2 wells, resuspended in 0.5ml unsupplemented RPMI-1640 and injected intraperitoneally. This immunogen was injected intraperitoneally into Balb/c mice at weekly intervals for 6 weeks.

c. Preparation of wheat germ lectin eluate

The wheat germ lectin eluate was prepared using first trimester placenta. Placental membrane proteins were solubilized by the addition of an 8mM solution of 3-[(cholamidopropyl)dimethylammonio]1-propanesulphonate (CHAPS) in 20mM Tris-HCl, pH 8.0 buffer with 0.02% (w/v) sodium azide, 5mM Ethylenediaminetetra-acetic acid (EDTA) and 1mM Phenylmethylsulphonylfluoride (PMSF) in the ratio of 1:10 (w/v). After stirring for 17 hours at 4°C the insoluble material was removed by centrifugation at 2000 g for fifteen minutes. Wheat Germ Agglutinin covalently linked to agarose beads (approx one ml of packed beads) was incubated batchwise, with stirring for 17 hours, with the solubilized placental preparation (approx 30 ml). The beads were then recovered by centrifugation at 200 g for five minutes and transferred to a column for washing with three volumes of 200 mM N-acetylglucosamine in homogenization buffer. For primary immunization in Balb/c mice, the extract was emulsified with Freund's Complete Adjuvant (1:1, v/v). One half ml of emulsion was injected subcutaneously at several sites into Balb/c mice. Three weeks following the primary immunization, three weekly boosts of 0.5ml of the extract alone were given intraperitoneally.

d. Fusion of immune mouse spleen cells with myeloma cells

Five days prior to the fusion procedure, mice were injected intraperitoneally with 0.5ml of Freund's Incomplete Adjuvant. The last boost was administered on the following day. Spleen cells from the immunized mice were fused with mouse myeloma P3x63.Ag8.653 cells. The fusion cell mixture was dispensed initially into 24 hour plates at a spleen cell concentration of 2 x 10^7 cells.ml and allowed to grow in the presence of RPMI 1640 (Flow

Laboratories) medium containing 10% (v/v) heat-inactivated fetal calf serum (Flow Laboratories), 1 x 10^{-7} aminopterin, 1.6 x 10^{-5}M thymidine, 2 mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humid atmosphere of 5% CO_2 in air for about 10 days. A few cells from each hybrid colony were then picked out and transferred to individual wells of other 24-well plates. To both minimise the probability of isolating colonies secreting MAbs which identified carbohydrate epitopes and facilitate the possible use of trophoblast-reactive MAbs in immuno-affinity gels, IgG secreting clones were identified using an antigen capture ELISA technique. Briefly, ELISA plates were coated with anti-mouse Ig and the culture supernatant from each hybrid colony added. After adequate incubation time and washing, an IgG class specific enzyme conjugated second antibody was added and developed with the appropriate enzyme substrate. All IgM secreting clones were discarded and the culture supernatants containing IgG or A were screened for antibody binding activity on frozen sections of first trimester placenta using an immunoperoxidase technique (see below). Cultures of interest were then cloned twice by the limiting dilution method and further expanded in culture.

e. Determination of the tissue specificity of monoclonal antibodies by immunoperoxidase staining of frozen sections

Sections of frozen tissue were cut at -20°C using an American Optical Cryostat. Five-µm thick sections were air-dried on chrome-alum gelatin-coated slides for 2 h. The tissue sections were incubated with cell culture supernatants containing antibodies for either 1-2 h at room temperature or overnight at 4 °C. Bound antibody was revealed by using biotin-labelled horse antibodies to mouse IgG followed by avidin-biotinylated peroxidase

complex (Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) (0.5 mg/ml) with 0.01% hydrogen peroxide in 20 mM imidazole buffer, pH 7.0, was the enzyme substrate. Sections were counterstained with Mayer's Haemalum. Cover slips were then mounted on air dried sections with DePeX.

For tissues containing substantial amounts of endogenous biotin such as liver and kidney, an indirect immunoperoxidase technique was used. An appropriate dilution of peroxidase-labelled goat anti-mouse IgG reduced the high background staining found with the avidin-peroxidase complex technique.

Human tissues were obtained as follows; first trimester placenta and decidua were obtained from elective pregnancy terminations, term placentas within 1 hr of delivery, and endometrium, myometrium , ovary, cervix, stomach, colon and rectum were obtained at surgery. The remainder were obtained within 6 h postmortem. Tissues were frozen in liquid nitrogen, then stored at -70°C for less than two months.

A monoclonal antibody (FDO114G) produced in the inventors' laboratory, which reacts with Type IV collagen, acted as a positive control on all tissues.

f. Selection of monoclonal antibodies to human trophoblast membrane protein.

The monoclonal antibodies described in this invention were selected on the basis of the following criteria:

1. They were of the IgG class.

2. The cell lines which secrete the respective antibodies are stable and fast growing in culture, continue to secrete the Mab concerned, can be stored frozen in liquid nitrogen and can be retrieved from the frozen state.

3. The monoclonal antibodies are strongly reactive with human villous syncytiotrophoblast and non-villous trophoblast on human placentas throughout pregnancy. They do not react with the mesenchymal cells of the placenta. FD0161G and FD066Q have very restricted reactivity with other human tissues, FD0338P does not react with any of a large panel of other human tissues tested. None react with human peripheral blood cells or serum components.

4. The protein antigens which these monoclonal antibodies define, viz. FD0161G/FD066Q protein and FD0338P protein are glycoproteins and hence the epitopes are most likely expressed on the surface membrane of trophoblast cells.

The application of these stringent criteria means that the Mabs selected have the optimal ability to extract trophoblast cells which circulate at very low frequency in maternal blood during pregnancy.

EXAMPLE 2

ISOLATION AND CONFIRMATION OF TROPHOBLAST CELLS FROM MATERNAL BLOOD SAMPLES

a. Preparation of antibody-coupled substrate

The source of each Mab is cell free culture supernatant from the respective cell line cultured in

standard conditions (RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal calf serum, 2 mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humid atmosphere of 5% CO_2 in air). The supernatant is collected from well grown cultures, centrifuged to remove cells and stored frozen at -20°C.

The substrate used is a preparation of commercially available, magnetic polystyrene beads precoated with sheep anti-mouse IgG (Fc fragment) serum (Dynabeads M-450, Dynal AS, Oslo, Norway).

One (1) ml of sterile supernatant is mixed with 20 million beads, and the tube rotated end-to-end on a mixer at room temperature. The coated beads are then stored at 4°C. Before use, the beads are washed three times in phosphate-buffered saline containing 1% (v/v) heat-inactivated fetal calf serum (2 min each wash). The beads are collected from the washing solution each time using a cobalt-samarium magnet. The tube is rested on the magnet for a few minutes; allowing the beads to settle on the inner surface of the tube resting on the magnet. The washing solution is aspirated off. The beads are finally resuspended in ice-cold RPMI 1640 culture medium containing L-Glutamine (2 mM) to give approximately 2.5 x 10^5 beads/ml.

b. Isolation of trophoblast cells from blood samples

A 25 ml sample of blood is collected from the antecubital vein of a pregnant woman into a syringe containing 10 IU of lithium heparin/ml whole blood. The blood is diluted ten fold with ice-cold RPMI 1640 medium containing 2 mM L-Glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml) and heparin (10 IU/ml), gently mixed, and kept at 4°C while the beads are being washed

as described above. The diluted blood and washed beads are mixed gently (4000 beads/ml whole blood i.e. 10^5 per 25 ml sample) and incubated at 4°C overnight. The beads, with trophoblast cells attached via the specific trophoblast reactive antibody, are removed using a cobalt-samarium magnet (Dynal AS). The large volume of the mixture may be processed continuously (by flow over the magnet) or batchwise to remove the beads. In the case of batchwise processing, the volume of the medium containing the beads is finally reduced to 0.4 ml through successive extraction and washing. At this stage, the bead-cell mixtures can either be prepared for DNA sequence amplification or placed into culture.

c. Method for detection of cells containing a Y chromosome

In order to verify that the cells isolated by the beads with bound Mab to trophoblast membrane protein were indeed, fetal in origin, a Y chromosome specific signal was employed to detect the Y chromosome in the isolated cells. The polymerase chain reaction (PCR) was used since the cells are present in very low numbers. The male specific signal has been obtained using a pair of DNA primers to a Y chromosome repeat sequence. The primer pair which was found to give satisfactory specificity and signal strength was designated WYR7 and WYR8. The size of the amplified DNA sequence is 124 base pairs (bp). The sequence of the primers are as follows:

WYR7 5' TGG GCT GGA ATG GAA AGG AAT CGA AAC 3'
WYR8 5' TCC ATT CGA TTC CAT TTT TTT CGA GAA 3'

The protocol used has been as follows:

1. Cells bound to magnetic beads were aliquoted into 0.5 ml Eppendorf tubes in saline. The volume of saline has been minimised although for 25 µl of PCR buffer

mixture, the reaction is tolerant of saline up to a volume of at least 10 µl.

2. Cells in saline have been lysed by heating in a waterbath at 100°C for 10 min. The cell suspension is covered by a layer of paraffin oil and the tube is capped during this process.

3. PCR buffer is then added (to 25 µl). The final concentration of regents in the reaction mixture are: 67 mM Tris-HCl (pH 8.8 at 25°C), 2.0 mM $MgCl_2$, 0.01% (w/v) gelatin, 10 mM β-mercaptoethanol, 16.6 mM ammonium sulfate, 17 µg/ml bovine serum albumin, 10% dimethyl sulfoxide, 0.1 mM deoxynucleotide triphosphates, 0.25 µg of each primer and 0.2 units of Taq DNA polymerase (Thermophilus aquaticus).

4. 30 cycles of PCR are then carried out as follows:

Denaturation step	94°C for 1 min
Annealing step	55°C for 1 min
Polymerase extension step	72°C for 1 min

Each round of amplification is the same except that (i) the first denaturing step is for a period of 6 min., (ii) extension time of the final cycle is 10 min.

5. The PCR product is electrophoresed in agarose or polyacrylamide gels by standard methods and visualized under ultraviolet light after the gel has been stained with ethidium bromide.

Trophoblast cells bound to magnetic beads have been accurately quantified by microscopy and a Y chromosome specific signal can be reproducibly obtained from only 6 male lymphocytes after 30 cycles of the PCR. An equivalent number of female cells taken through the same PCR yields no detectable signal or an exceedingly

weak signal readily distinguished from that of male cells.

The signals obtained from control trophoblast cells derived from a full-term placenta of a male child and CVS confirmed male and female fetal cells isolated by Mab coated beads are demonstrated in Figure 1. The inventor's laboratory has processed eleven blood samples from pregnant women undergoing chorionic villous sampling biopsy. The predicted sex of the fetus was confirmed in eleven out of eleven cases ($P < 0.0005$).

d. Culture of isolated fetal trophoblast cells

Trophoblast cells, with beads attached are placed into 48 well plastic culture dishes with coverslips in the base of the dishes and covered with a complex culture medium comprising a 1:1:1 mixture of (a) culture supernatant from human decidual cells obtained from elective termination of early pregnancy, (b) culture supernatant from human granulosa cells (obtained during the procedure of egg collection in an In Vitro fertilization programme) and (c) standard culture medium RPMI 1640 containing 10% (v/v) normal human female serum, 2 mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin) at 37°C in a humid atmosphere of 5% CO_2 in air. After 14-21 days culture, the trophoblast cell cultures have reached sufficient cell density to perform chromosome studies. This is done by standard techniques performed in cytogenetic diagnosis. The culture supernatants (a) and (b) (above) were obtained from cultures of decidual cells and granulosa cells in standard medium; the culture supernatant was collected, centrifuged to remove cells and stored at -20°C.

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CLAIMS:

1. A method for the isolation of trophoblast cells from a blood sample of a pregnant mammal which method comprises contacting said blood sample with a binding-effective amount of an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells for a time and under conditions sufficient for said antibody to bind to target cells and then separating cells bound by said antibody from said sample.

2. The method according to claim 1 wherein said mammal is a human female.

3. The method according to claim 1 or 2 wherein the antibody is specific for epitope(s) of a trophoblast membrane protein resident on villous syncytiotrophoblast and non-villous cytotrophoblast cells.

4. The method according to claim 3 wherein the antibody is specific for epitope(s) of a trophoblast membrane protein resident on villous syncytiotrophoblast and non-villous cytotrophoblast cells but which are not present on any other blood-borne cells or any serum component of maternal or fetal origin.

5. The method according to claim 4 wherein the trophoblast membrane protein precursors have molecular weights on sodium dodecyl-sulphate polyacrylamide gel electrophoresis of from about 30 kDa to about 70 kDa.

6. The method according to claim 4 wherein the protein is a surface-expressed trophoblast membrane protein having a molecular weight on sodium dodecyl-sulphate polyacrylamide gel electrophoresis of about 60 kDa to about 70 kDa.

7. The method according to claim 4 wherein the protein is a glycoprotein and has no N-linked high mannose chains but has between 3 and 6 O-linked and between 5 and 10 complex N-linked oligosaccharide chains per molecule or in which the glycoprotein has no N-linked but between 3 and 6 O-linked oligosaccharide chains per molecule.

8. The method according to claim 7 wherein the trophoblast membrane protein has a molecular weight of about 43kDa, an N-terminal sequence of Thr-Gly-Trp-Ser-His-Leu-Val-Thr-Gly-Ala-Gly-Gly-Phe-Leu-Gly-Gln-(Y)-Ile-(Y)-(Y)-Leu-(Y)-Val-Lys-(Y), and a partial internal sequence of -Phe-(X)-Leu-Arg-Leu-Glu-Ser-Arg-(X)-Ser-Phe-Pro-Leu-(Y)-(X)-Met-Tyr-(X)-Ile-, wherein X and Y are as hereinbefore defined.

9. The method according to any one of the preceding claims wherein the antibody is either a monoclonal antibody or a polyclonal antibody or a combination thereof.

10. The method according to claim 9 wherein the antibody is FD0161G or FD066Q or FD0338P and/or any combinations thereof.

11. A method for obtaining fetal genetic and/or biochemical information in a pregnant mammal which method comprises isolating a blood sample from said pregnant mammal and contacting said blood sample with a binding-effective amount of an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells for a time and under conditions sufficient for said antibody to bind to target cells and then separating cells bound by said antibody from said sample and

obtaining from the isolated cells genetic and/or biochemical information.

12. The method according to claim 11 wherein said mammal is a human.

13. The method according to any one of claims 11 to 12 wherein the antibody is specific for epitope(s) of a trophoblast membrane protein resident on villous syncytiotrophoblast and non-villous cytotrophoblast cells.

14. The method according to claim 13 wherein the antibody is specific for epitope(s) of a trophoblast membrane protein resident on villous syncytiotrophoblast and non-villous cytotrophoblast cells but which are not present on any other blood-borne cells or any serum component of maternal or fetal origin.

15. The method according to claim 14 wherein the trophoblast membrane protein precursors have molecular weights on sodium dodecyl-sulphate polyacrylamide gel electrophoresis of from about 30 kDa to about 70 kDa.

16. The method according to claim 13 wherein the protein is a surface-expressed trophoblast membrane protein having a molecular weight on sodium dodecyl-sulphate polyacrylamide gel electrophoresis of about 60 kDa to about 70 kDa.

17. The method according to claim 13 wherein the protein is a glycoprotein and has no N-linked high mannose chains but has between 3 and 6 O-linked and between 5 and 10 complex N-linked oligosaccharide chains per molecule or in which the glycoprotein has no N-linked

but between 3 and 6 O-linked oligosaccharide chains per molecule.

18. The method according to claim 13 wherein the trophoblast membrane protein has a molecular weight of about 43KDa, an N-terminal sequence of Thr-Gly-Trp-Ser-His-Leu-Val-Thr-Gly-Ala-Gly-Gly-Phe-Leu-Gly-Gln-(Y)-Ile-(Y)-(Y)-Leu-(Y)-Val-Lys-(Y), and a partial internal sequence of -Phe-(X)-Leu-Arg-Leu-Glu-Ser-Arg-(X)-Ser-Phe-Pro-Leu-(Y)-(X)-Met-Tyr-(X)-Ile-, wherein X and Y are hereinbefore defined.

19. The method according to any one of claims 9 to 14 wherein the antibody is either a monoclonal antibody or a polyclonal antibody or a combination thereof.

20. The method according to claim 19 wherein the antibody is FDO161G or FDO66Q or FDO338P and/or any combinations thereof.

21. The method according to any one of the preceding claims wherein the antibody is first bound to a solid substrate.

22. A method according to claim 21 wherein the solid substrate comprises magnetic beads.

23. The method according to claim 22 wherein the substrate comprises magnetic polystyrene beads precoated with sheep anti-mouse IgG (Fc Fragment) serum.

24. A method according to claim 22 wherein the separation of cells bound by antibody from the sample comprises the step of continuously passing the sample in a flow past a magnet.

25. The method according to any one of claims 1 to 20 wherein the antibody is first conjugated to a fluorescent label.

26. The method according to claim 25, wherein the fluorescent label is one suitable for use in fluorescence-activated cell sorting.

27. Homogeneous or near homogeneous antigenic FD0161G/FD066Q protein, or any combination thereof.

28. Homogeneous or near homogeneous antigenic FD0338P protein.

29. A kit for the isolation of trophoblast cells from a blood sample of a pregnant mammal and optionally for obtaining genetic and/or biochemical information about said cells comprising in compartmental form a first container adapted to contain an antibody specific for villous syncytiotrophoblast and non-villous cytotrophoblast cells; optionally a second container adapted to receive and contain a blood sample from said pregnant mammal; and optionally a third container adapted to contain a means for obtaining genetic and/or biochemical information from the isolated trophoblast cells.

30. The kit according to claim 29 wherein the antibody in the first container is either a monoclonal antibody or a polyclonal antibody or a combination thereof.

31. The kit according to claim 30 wherein the antibody is FD0161G or FD066Q or FD0338P and/or any combinations thereof.

32. The kit according to claim 29 wherein the means for obtaining genetic and/or biochemical information includes means for culturing the isolated trophoblast cells.

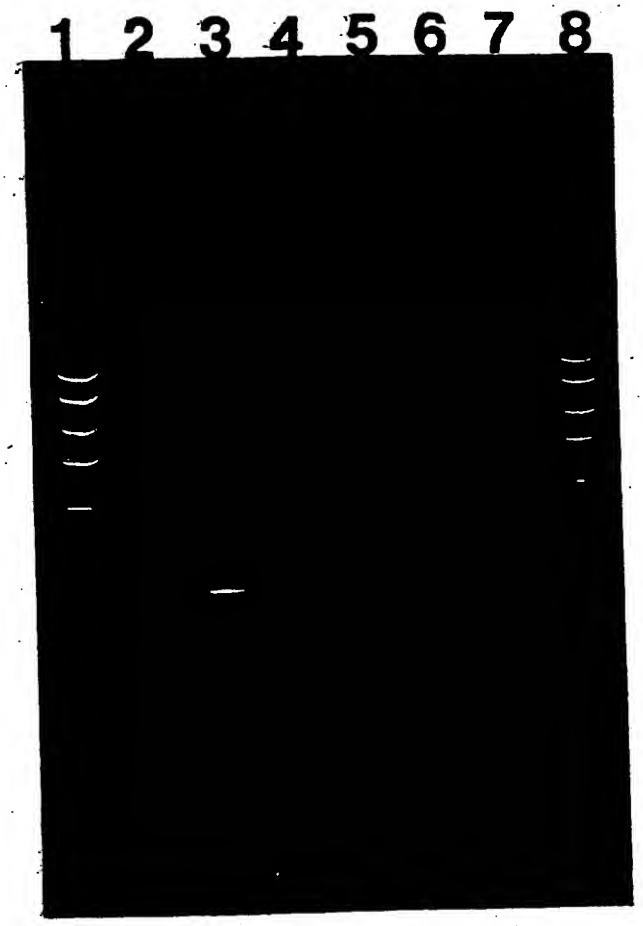
33. The kit according to claim 29 wherein the means for obtaining genetic and/or biochemical information includes DNA probes or primers.

34. The kit according to claim 29 wherein the antibody is bound to a solid substrate.

35. The kit according to claim 34 wherein the substrate comprises magnetic polystyrene beads precoated with sheep anti-mouse IgG (Fc Fragment) serum.

36. The kit according to claim 29 wherein the antibody is conjugated to a fluorescent label.

37. The kit according to claim 36 wherein the fluorescent label is one suitable for use in fluorescence-activated cell sorting.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INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00517

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] G01N 33/53, C07K 15/14, C07K 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	G01N 33/53, C07K 15/14, C07K 15/12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU IPC : As above
Chemical Abstracts; keywords : Trophoblast, syncytiophoblast, cytotrophoblast

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	Biochemistry, Vol 25 634-640, 1986 Sen-Majumdar et al "A specific Antibody to a New Peptide Growth Factor from Human Placenta : Immunocytochemical Studies on Its Location and Biosynthesis see particularly abstract.	1-6, 9-10
X	Chemical Abstracts, Vol 102, no. 5 issued February 4, 1985 (Columbus, Ohio, U.S.A.), Hattori, Atsuo et al, 'Study on immunohistochemical localisation of alkaline phosphatase by the monoclonal antibodies' see page 417, column 2, the abstract no. 44039 Sapporo Igaku Zacchi 1984, 53(5), 611-23 (Japan).	1-6, 9-10

CONTINUED

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6 March 1990 (06.03.90)	26 MAR 1990
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	A.W. BESTOW

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Chemical Abstracts, Vol 103 no. 12 issued September 23, 1985, (Columbus, Ohio (U.S.A), Butterworth, Bridget H et al, 'Human cytotrophoblast populations studied by monoclonal antibodies using single and double biotin-avidin-peroxidase immunocytochemistry' see page 346, column 1 the abstract no. 192486 J. Histochem. Cytochem. 1985 33(10), 97783 (England).	1-37

V. [] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[] Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.[] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[] The additional search fees were accompanied by applicant's protest.

[] No protest accompanied the payment of additional search fees.